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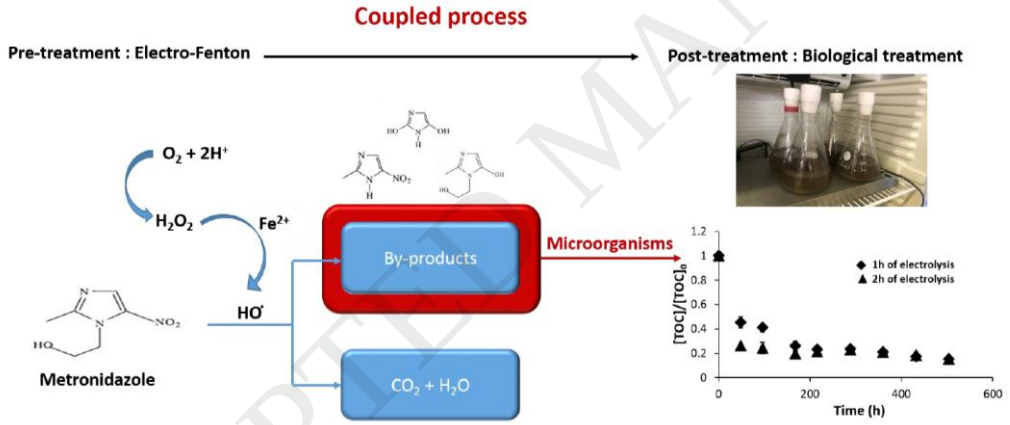
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Metronidazole removal by means of a combined system coupling an electro-Fenton process and a conventional biological treatment: by-products monitoring and performance enhancement

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Graphical abstract



Highlights :

- Biological treatment of MTZ led to 22% of degradation after 21 days of treatment
- The application of EF led to total MTZ degradation with low level of mineralization
- The biodegradability increased from 0 to 0.46 for the BOD5 on COD ratio
- By-products were identified by UPLC-MS/MS and a degradation pathway was proposed.
- Overall removal yield of the MTZ effluent during the combined process was 87.4%

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Abstract

In order to mineralize Metronidazole (MTZ), a process coupling an electro-Fenton pretreatment and a biological degradation was implemented. A mono-compartment batch reactor containing a carbon-felt cathode and a platinum anode was employed to carry out the electro-Fenton pretreatment of MTZ. A total degradation of MTZ (100 $mg.L^{-1}$) was observed at 0.07 $mA.cm^{-2}$ after only 20 min of electrolysis. Yet, after 1 and 2 hours of electrolysis, the mineralization level remained low (16.2% and 32 % respectively), guaranteeing a significant residual organic content for further biological treatment. LC-MS/MS was used to determine the intermediates by-products and hence to propose a plausible degradation pathway. An increase from 0 to 0.44 and 0.6 for 1 and 2 hours of electrolysis was observed for the BOD_5/COD ratio. Thus, from 1 hour of electro-Fenton pretreatment, the electrolysis by-products were considered biodegradable. A biological treatment of the electrolysis by-products after 1 and 2 h was then realized. The mineralization yields reached very close values, about 84% for 1 and 2 h of electrolysis after 504 hours of biological treatment, namely close to 89% for the overall process, showing the pertinence of the proposed coupled process.

Keywords : Biological treatment; activated sludge; Electro-Fenton process; Metronidazole; by-products monitoring

1. Introduction

Because of high levels of consumption, long persistence in the environment and a very varied composition, antibiotics are considered as very critical water pollutants. They are clearly detected in sewage water even after treatment because of their recalcitrance [1]. The pollution generated by pharmaceutical drugs are originated from pharmaceutical industries emission, domestic wastes and hospital effluents [2–4]. Indeed, from the French ministry of the Ecology and from the Sustainable-development division of the Ecology ministry [5], one of the three major sources of water pollution is known to be industrial discharge and its relative part is respectively 15%.

To eliminate the pharmaceutical drugs from water, some processes such as adsorption on activated carbon in aqueous phase [6–8] are studied. This class of processes, although efficient, relies on a transfer of pollution from the aqueous phase to the solid phase and concerns low

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concentrated effluents. Among the destructive methods, biological treatment has been also tested, but appears not really efficient for such biorecalcitrant compounds and requires a long time for its completion [9,10]. Advanced oxidation processes AOP, based on a non-selective generation of powerful actives species such as ˚OH, have shown their efficiency for the degradation of several organic molecules [1] and for concentrated effluents. However, their employment for a complete mineralization is generally pricey and requires additional energy and consumption reagents [11].

For concentrated effluents, to combine the advantages of both kind of processes, their coupling have been proposed, namely the use of AOP as pre-treatment or post-treatment and biological treatment. The order of the coupling of the processes depends on the effluent load in non-biodegradable compound [11]. If the effluent contains a large amount of biodegradable compounds with low concentrations of recalcitrant compounds, a biological treatment is applied in pretreatment in order to remove all the biodegradable part of the effluent and subsequently the AOP is applied to remove the refractory part [12]. However, if the effluent contains toxic molecules for microorganisms and / or have biodegradability below 0.4, the AOP will take the place upstream. In this case, the aim of the AOP is to improve the biodegradability of the organic pollutant or to reduce its toxicity, thus enabling relatively inexpensive subsequent biological treatment [11–14]. Oller et al, reported in their review the interest and the efficiency of a combined process AOP-Biological treatment to treat such type of effluent, containing recalcitrant contaminants [11]. Among AOP, Electrochemical Advanced Oxidation Processes (EAOPs) are considered as ones of the most commonly used AOP during coupled processes, since they are characterized by several advantages, such as high efficiency, moderate operating costs, facility of automation and compatibility with environmental applications [15,16]. Ganzenko et al, confirmed the effectiveness of the coupling processs (EAOP and biological treatment) compared to the individual treatment [12].

Being among the most efficient EAOPs, Electro-Fenton (EF) process is applied as a pre-treatment in the present study followed by a conventional biological treatment. The EF process is considered as an environmentally clean treatment [17]. It allows to minimize the use of the reagent and the formation of ferric hydroxides. This process is based on the *in-situ* generation of H_2O_2 by reducing the dissolved oxygen (Eq. (1)). The generated H_2O_2 reacts with the dissolved Fe (II) to produce powerful radicals ˚OH and Fe (III) (Eq. (2)). Fe(II) was regenerated by the cathodic reduction of Fe(III) (Eq. (3)) [18]. According to Olvera-Vargas et al, this type of coupling is called bio-electro-Fenton (Bio-EF), which combines the high oxidation power

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of EF and the profitability of biological treatment for the removal of refractory compounds [16].

$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$	*E° = 0.69 V/SHE*	(1)
$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$	*(Fenton's reaction)*	(2)
$Fe^{3+} + e^- \rightarrow Fe^{2+}$	*E° = 0.77V/ SHE*	(3)

This study focuses therefore on the application of a coupled process (electro-Fenton/ biological treatment) to treat an effluent loaded with a recalcitrant pharmaceutical drug, Metronidazole (MTZ). This antibacterial and anti-inflammatory antibiotic is detected in treated sewage water [19] and is characterized by its non-biodegradability, its high solubility in water, its toxicity to aquatic system, its potential mutagenicity, and its carcinogenicity [20–22]. The adsorption of metronidazole on granular activated carbon followed a Langmuir model with a maximal sorption capacity of 110.64 $mg.g^{-1}$[23] but for a total MTZ removal, a post treatment was necessary. Degradation processes such as photocatalytic ozonation [24], sono-Fenton [25], anodic oxidation [26], direct photolyse, Fenton and photo-Fenton [27] were tested with degradation yields up to 85%.

Métronidazole removal was also studied by photo-electro-Fenton and mineralization yield reached 53% [28].

In a previous paper on the mineralization of metronidazole, the objective was to optimize the electro-Fenton process and to verify the feasibility of coupling electro-Fenton with a biological treatment [29]. This study aims to improve the mineralization rate of the MTZ by applying a process coupling electro-Fenton and biological treatment. In this sense, a follow-up of the mineralization of the MTZ, the biodegradability of the by-products of electrolysis and the identification of by-products during the electro-Fenton process and the biological treatment were examined to understand the degradation mechanism of MTZ

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2. Materials and methods

2.1. Chemicals and materials

The target pollutant, metronidazole, MTZ, (2-methyl-5-nitroimidazole-1-ethanol), and the by-product of MTZ, MAA (2-methyl-5-nitroimidazole-1-acetic acid), were obtained from Sigma–Aldrich. $FeSO_4 \cdot 7H_2O$ (purity 99 %) and Na_2SO_4 (purity 99 %) were purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Ultrapure water (Millipore Elga) was used to prepare all solutions.

2.2. Electrochemical Apparatus and Procedures

An electrochemical batch reactor containing 250 mL of solutions was employed in all experiments carried out at ambient temperature (20°C). An ammeter power supply (Microsonic systems, Microlab MX 20V-2A, France) was used to apply the current intensity at 0.07 $mA.cm^{-2}$. A tri-dimensional piece of graphite felt of 42 cm^3 (geometrical volume) (Le Carbone Lorraine RVG 4000 Mersen, Paris la Défense, France) was the working electrode (cathode) positioned on the inner wall of the cell; while cylindrical platinum (32 cm^2) was used and placed at the center of the electrochemical reactor as an anode. The latter was surrounded by the cathode to get a good potential distribution. 10 min prior the electrolysis and throughout the experiments, compressed air was bubbled into the solution (0.5 $L.min^{-1}$) in order to reach a stationary O_2 concentration. Just before the start of the electrolysis, a catalytic quantity of $FeSO_4 \cdot 7H_2O$ was added into the cell (0.1 $mmol.L^{-1}$). The ionic strength was maintained constant by the addition of 50 $mmol.L^{-1}$ Na_2SO_4.

2.3. Preparation of activated sludge

Activated Sludge used in this study was collected from the municipal wastewater treatment plant (Rennes Beaurade, France). The activated sludge was maintained in an 8 L reactor in the laboratory. For growth and conservation, the activated sludge was cultivated under oxygen flow on the following mineral medium ($g.L^{-1}$) : peptone, 0.64 ; K_2HPO_4, 0.11 ; NH_4Cl, 15.2 ; CH_3COONa, 140 ; and some drops per month of Viandox as a supplementary source of carbone. Prior to the inoculation, in order to avoid any residual carbon and mineral source other than those contained in the culture media, the active sludge was washed three times with tap water and two times with ultra-pure water.

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2.4. Determination of the biological oxygen demand (BOD_5)

BOD_5 measurements were carried out in Oxitop IS6 (from WTW, Alès, France) at 20°C, pH 7 and in obscurity during 5 days in order to evaluate the biodegradability of MTZ molecule and its by-products. For inoculation, an initial microbial concentration of 0.05 $g.L^{-1}$ was needed. The mineral basis, nitrification inhibitor, and buffer solution presented in the table 1 were used for all experiments.

A control sample containing biodegradable compounds, namely glutamic acid (150 $mg.L^{-1}$) and glucose (150 $mg.L^{-1}$) was carried out to check the viability of the activated sludge. A blank solution, containing water (without a source of carbon) was performed to deduce the biological oxygen demand corresponding to the endogenous respiration.

2.5. Biological treatment

Cultures were performed in duplicate for 21 days at 25°C and in 500 mL Erlenmeyer flasks, magnetically stirred (200 rpm), closed with a cellulose cap to guarantee oxygenation and loaded with 200 mL of non-treated Metronidazole or Metronidazole solutions electrolyzed by EF for 1 or 2 hours. An initial activated sludge concentration of 0.5 g L^{-1} was inoculated and the following minerals were used for all experiments ($mg.L^{-1}$) : K_2HPO_4, 208 ; KH_2PO_4, 85 ; $Na_2HPO_4.2H_2O$, 154.4 ; $MgSO_4.7H_2O$, 22.6 ; $CaCl_2$, 27.6 ; $FeCl_3.6H_2O$, 0.26 ; NH_4Cl, 75.[30]. The pH was then fixed to 7.0 with NaOH solution (2 $mmol.L^{-1}$). Samples were taken regularly for TOC and HPLC measurements and filtered on 0.45 µm Chromafil® Xtra PET –45/25.

2.6. Biosorption tests

A protocol similar to that of the biological treatment was used, except that culture medium was inoculated with two types of inocula: activated sludge and sludge inactivated by autoclaving at 120°C for 20 min in order to avoid the phenomenon of biodegradation and evaluate only the biosorption. Metronidazole and its electrolysis by-products were inoculated with the two types of biomass (active and inactive) at a concentration of 0.5 $g.L^{-1}$ in the presence of the minerals used in the biological treatment at 25°C with a stirring of 200 rpm; regular samples were taken during 180 min. The samples taken were then analyzed by TOC-meter. Experiments were performed in 500 mL Erlenmeyer flasks.

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2.7. Phytotoxicity test

The phytotoxicity test was carried out using seeds of garden cress (*Lepidium Sativum*). A non-toxic compound must have a germination index (GI) greater than 80 %. For its determination, the preparation of 10 Petri dishes containing filter paper, moisten with 5 ml of MTZ or MAA was carried out. Then, the deposition of 10 seeds of garden cress on the filter paper was realized. The petri dishes were stored in the dark at 25°C for 48 hours. The same steps must be carried out for the control, with Petri dishes containing only tap water.

%Germination Index = % Germination x % Root growth

$$\% \text{ germination} = \frac{\text{Number of germinated seeds for the test}}{\text{Number of germinated seed for control}}\ x\ 100$$

$$\% \text{ Root Growth} = \frac{\text{Size of roots of germinated seeds for test}}{\text{Size of roots of germinated seeds for control}} x100$$

2.8. Analytical procedures

2.8.1. LC-MS/MS method

The identification of the different by-products from electrolysis as well as from the biological treatment was determined by Liquid Chromatography coupled to Mass Spectrometry (UPLC-MS/MS). The analytes was separated by a Waters Acquity UPLC system (Waters, Manchester, UK). The system consisted of an Acquity UPLC binary solvent manager, an Acquity UPLC sample manager and an Acquity UPLC column heater equipped with a Waters Acquity UPLC BEH C-18 column (2.1 mm × 100 mm; 1.7 µm) (Milford, MA, USA). Two mobile phases A and B were used to carry out the UPLC elution gradient. The first phase was performed in ultra-pure water with 0.1 % formic acid; the second was performed in acetonitrile with 0.1 % formic acid. In order to separate the analytes on the column, the process was carried out with a gradient of phase A/phase B at the flow rate of 0.4 mL.min^{-1}. The starting eluent composition consisted of 0 % A/100 % B for 1 min; then the proportion of solvent increased linearly to reach 100 % in 8 min.

Waters Micromass Quattro Premier (Waters Corporation, Manchester, UK) triple quadrupole mass spectrometer was used to detect the separated compounds. The mass spectrometer was constituted of an electrospray source in positive ionization mode with a cone potential of 30 V. The ionization source conditions were: capillary voltage of 3.0 kV and source temperature of

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350°C. The cone and desolvation gas flows were 50 $L.h^{-1}$ and 750 $L.h^{-1}$ respectively. These values were obtained from a nitrogen source, the Peak Scientific NM30LA (Peak Scientific, Inchinann, United Kingdom). High-purity argon (99.99 %, Air Liquid, Paris, France) was used as collision gas and was regulated at 0.1 mL min^{-1}. Collision energy of 25 eV was fixed during the implementation of analyses in full scan and daughter scan modes. Spectra were acquired between 80 and 500 mz^{-1} and the data were treated with Micromass Mass-Lynx 4.1 software.

2.8.2. Ionic chromatography (CI)

DIONEX DX120 ion chromatography was used to identify the generated carboxylic acids and to quantify nitrite and nitrate ions. The chromatograph was equipped with a conductivity detector, using an anion exchange column AS19 (4 x 250 mm) as the stationary phase, and a solution of KOH (12 $mol.L^{-1}$) in water as the mobile phase. A gradient elution mode was used to perform all analyses. It started with 10 $mmol.L^{-1}$ of KOH during 10 min then increased linearly to 45 $mmol.L^{-1}$ from 10 to 25 min and finally kept at 45 $mmol.L^{-1}$ of KOH from 25 to 35 min. The flow rate was set at 1 mL min^{-1}.

2.8.3. High performance liquid chromatography (HPLC)

The evolution of MTZ concentrations was monitored by HPLC using Waters 996 system equipped with Waters 996 PDA (Photodiode Array Detector) and Waters 600LCD Pump. Waters C-18, (4.6 x 250 mm x 5 µm) reversed-phase column was used to achieve the separation. A mixture of acetonitrile/ ultra-pure water (20/80, v/v) with 0.1 % formic acid was used as eluent and the flow rate was 1 $mL.min^{-1}$. The detection of Metronidazole was carried out at 318 nm and the retention time was 4.3 min.

2.8.4. Total organic carbon (TOC) and total nitrogen (TN) measurements

TOC and TN were measured by means of a TOC-$V_{CPH/CPG}$ Total Organic Analyzer Schimadzu. Organic Carbon compounds were combusted and converted to CO_2 which was detected by a non-dispersive Infra-Red Detector (NDIR). Dissolved Nitrogen compounds were combusted and converted to NO which was then mixed with Ozone chemiluminescence for detection by a photomultiplier [17]. The mineralization current efficiency (MCE) was calculated from

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2.8.5. Ammonium species quantification

The monitoring of ammonium species (NH_4^+) was realized via the spectrophotometric Nessler method at 420 nm [31].

2.8.6. Chemical Oxygen Demand (COD) measurements

COD was measured by Nanocolor® CSB 160 kits from Macherey-Nagel (Düren, Germany) by a Nanocolor photometer. The oxidation is carried out by potassium dichromate ($K_2Cr_2O_7$) in a strong acid medium (H_2SO_4) and at a temperature of 160 ° C for 30 minutes.

3. Results and discussion

3.1. Pretreatment of MTZ by the electro-Fenton process.

The effects of the electro-Fenton process on Metronidazole removal and mineralization are displayed in Fig 1. The electrolysis was carried out at 0.07 $mA.cm^{-2}$ with 0.1 $mmol.L^{-1}$ of Fe (II) and 0.05 $mol.L^{-1}$ of Na_2SO_4 with and initial amount of MTZ of 100 $mg.L^{-1}$. These conditions were previously determined to be optimal in a previous study [29]. After 20 min of electrolysis, a total degradation of MTZ was observed with an apparent kinetic (kapp) of 0.24 min^{-1}; while mineralization remained limited to 32 % after 120 min with a mineralization current efficiency (MCE) [18] of 26.3 %. This value was calculated from the following equation: $C_6H_9N_3O_3 + 18\ H_2O \rightarrow 6CO_2 + 3NO_3^- + 45H^+ + 42e^-$. This low mineralization rate is related to the formation of $^{\cdot}OH$ which may be insufficient to completely mineralize MTZ. The recalcitrance of MTZ for mineralization was previously observed during electro-Fenton process [28], using nano-scale zero-valent iron [32] and electro-Fenton with a Ce/SnO_2-Sb coated titanium anode [33]

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3.2. Identification of the intermediates products

Table 2 summarizes the various compounds detected via LC-MS/MS during the electro-Fenton treatment (from T0 min to T120 min) of 100 $mg.L^{-1}$ of metronidazole at 0.07 $mA.cm^{-2}$. These results showed that the removal of metronidazole is complex leading to various heterocyclic intermediates.

Based on the identification shown in the Table 2, a pathway for the degradation of metronidazole by hydroxyl radicals can be suggested (Fig.2). Hydroxyl radicals can attack metronidazole by different ways (Fig. 2): N-denitration leading to the release of a nitrate group, oxidation of N-ethanol to N-acetic acid, split of the N-ethanol group and hydroxylation that consists of one OH group substitution. The detection of the by-products of MTZ degradation via radical attack have been reported in various studies [28,34–37]. In the study of Cheng et al., the molecules 2 and 3 were detected using nanoscale zero-valent iron particles to remove the metronidazole [36], it can also be noted that the molecules 1,4, 5 and 7 were detected in the study of Pérez et al. on the degradation of metronidazole by solar photoelectro-Fenton [28].

It can be suggested that the degradation was initiated by the formation of compound 1 after the oxidation of the N-ethanol group or by the formation of compound 2 after an N-denitration of MTZ. Compound 2 can produce compound 5 after hydroxylation and compound 6 after oxidation of the lateral N-ethanol group. Compound 4 can be obtained by hydroxylation of compound 1 or oxidation of the lateral N-ethanol group of compound 5. Hydroxyl radical attack may cause the split of the N-acetic acid group which produced in our case compounds 7, 9 and 10. Substitution of the OH group on the imidazole ring facilitated the opening of the ring and produced small aliphatic carboxylic acids such as formic acid and oxalic acid. These acids were detected using ion chromatography (Table 2) and highlighted the mineralization of the target compound.

3.3. Evolution of the generated inorganic ions

MTZ contains two nitrogen atoms in its ring and a substituted nitrite group on the ring. Fig.3 illustrates time-courses of the quantification of nitrate and nitrite assayed by ion chromatography, ammonium by the Nessler method and total nitrogen by TOC-meter during electrolysis. There was no formation of nitrite; while nitrate formation was detected since 60 min of electrolysis, that resulted most likely from N-denitration, with a liberation of a nitrate group to obtain, for example, compounds 4, 5, 6, 9 detected by LC-MS/MS (Table 2). After 2

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hours of electrolysis, its concentration reached 0.3 mmol.L^{-1}. It is also observed that the generation of ammonium ions began after 60 min of electrolysis; they resulted from the radical attack of the nitrogen atoms contained in the metronidazole cycle. The ammonium concentration reached 0.45 mmol.L^{-1} at the end of the electrolysis. 38.7 % of the nitrogen atoms of the target molecule were transformed into ammonium, while 28.0 % were transformed into nitrates after 120 min of electrolysis. The percentage of undetected nitrogen may be related to the presence of nitrogen by-products in solution (Table 2). However, the total nitrogen concentration was constant throughout the treatment, which shows the absence of nitrogen evolution (N_2). Similar results were obtained by Annabi et al. on the production of nitrogen ions during electro-Fenton treatment of Enoxacin. Indeed, no nitrite NO_2^- anions were detected; 24 % and 5 % of the nitrogen atoms were transformed into ammonium and into nitrates after 120 min of electrolysis respectively and the total nitrogen concentration was constant [17].

3.4. MTZ biodegradability study.

The BOD_5 on COD ratio assesses the biodegradability of a solution. The solution is considered as biodegradable if the ratio is greater than or equal to 0.4 [38]. The objective of this estimation is to determine the feasibility of a biological treatment for MTZ mineralization subsequently to the electro-Fenton pretreatment.

Fig.4 displays the biodegradability in terms of time of electrolysis for MTZ under the optimal conditions of the electro-Fenton process. The non-biodegradability of the MTZ before electrolysis can be noted. After 30 min of electrolysis, a total degradation of MTZ was reached (Fig.1); while the solution remained poorly biodegradable with a ratio of 0.24 (<0.4). The first by-products of electrolysis were therefore relatively resistant to microorganisms since their chemical structures are very close to the target molecule. However, after 60 min electrolysis the solution became biodegradable, 0.46, namely above the threshold level (0.4). It can be inferred that after 60 min, an important part of the recalcitrant by-products have been oxidized. An increase of biodegradability was observed after 120 min of electrolysis, with a BOD_5 on COD ratio of 0.7. The choice of electrolysis duration can be posed for the EF-Bio coupling. Indeed, 60 min electrolysis was more economic from an energetic point of view, while 120 min electrolysis provided a higher level of biodegradability.

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3.5. Biosorption tests for MTZ and its by-products

Biosorption is defined as the adsorption of organic molecules on the wall of microorganisms. This accumulation is caused by a complex mechanism [39]. It is considered as a fast phenomenon, which occurs within the first few hours.

In order to evaluate biosorption phenomenon, it is possible to use activated sludge according to some authors [19,40,41]. Using living microorganisms, two phenomena can occur: biosorption or / and biodegradation. In order to avoid possible biodegradation even if a short experimental time was considered, inactivated sludge was used to determine the biosorption of MTZ and the electrolyzed by-products (Fig.5). The adsorption capacity of inactive microorganisms is higher than that of active microorganisms [42], due to the increase of the adsorption surface. Since the TOC value of the inactive microorganisms was stable (Fig.5), there was no biosorption of MTZ and its by-products on inactivated sludge. It can be then supposed that this phenomenon can be considered as negligible with activated sludge. It was confirmed for MTZ, since constant TOC values were also observed (Fig.5); while a slight decrease was observed for the biosorption of the by-products on activated sludge. This may be related to the biodegradation of the highly biodegradable compounds obtained after the electro-Fenton pretreatment, as confirmed thereafter.

3.6. Biological treatment of MTZ

A biological treatment was carried out first with non-treated MTZ as a sole source of carbon. Fig.6 shows that TOC values remained nearly constant during 504 hours of culture; the lack of mineralization was expected and in agreement with the low value obtained for the BOD_5 on COD ratio. However, the concentration of metronidazole decreased slightly. In fact, it can be assumed that at the beginning of culture, microorganisms were not able to assimilate the metronidazole, a clear decrease of 22 % was only observed from 216 h of culture; this decrease was also detected by Saidi et al [19], who observed a degradation of 15 % after 10 days of MTZ treatment by activated sludge.

Fig. 7 shows that the decrease in MTZ concentration was accompanied by the progressive production of a by-product. LC-MS/MS analyzes allowed to identify it as 2-methyl-5-

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nitroimidazole-1-acetic acid (MAA), which was also detected during the electro-Fenton pretreatment (Table 2). It was also detected as a by-product of MTZ by solar photo-electro-Fenton treatment [28], by photo-Fenton treatment [35]. Furthermore, MAA was detected as one of the MTZ metabolites formed by the action of the cytochrome P-450 system present in the liver [43]. The MAA activity against anaerobes is negligible compared to MTZ activity [44]; while it was shown in the present study that the phytotoxicity of MAA is higher than MTZ with a percentage of germination index of 65 and 74 respectively (Fig. 8). The constant concentration of total organic carbon (Fig. 6) and the formation of MAA (Fig. 7) indicated that MTZ had not undergone mineralization (Conversion to CO_2 and H_2O) but biotransformation (transformation of compound by the enzymatic activity of microorganisms to form another compound) and that microorganisms did not use MAA as a source of carbon or energy. At the end of the activated sludge treatment at 504 h, the TOC amount corresponded to 78 % of untreated MTZ and 22 % of produced MAA. These compounds were accumulated in the medium without being able to be eliminated, confirming the limits of the biological treatment with activated sludge and the need for a pretreatment.

3.7. Biological treatment of MTZ by-products

In order to confirm the efficiency of the coupled process, electro-Fenton and biological treatment, an activated sludge culture of by-product solutions after 1 or 2 h of electrolysis was carried out. The TOC was checked for 504 h and its monitoring for the pretreated solutions is shown in Fig. 9. Unlike pure MTZ, mineralization yields were clearly improved during the biological treatment of electrolyzed solutions, highlighting the pertinence of an electro-Fenton pretreatment of MTZ prior to a biological process.

Indeed, mineralization yield after 168 h increased to 74 and 80 % for the solutions pretreated during of 1 h and 2 h, respectively. The TOC evolution showed that the major part of the by-products from electrolysis was easily biodegradable. During the first 120 h of biological pretreatment, it can be observed that the mineralization of the pretreated solution for 2 h was higher than for 1 h of pretreatment; it was in agreement with the biodegradability results (Fig. 4). Nevertheless, no significant increase was detected from 168 h to 288 h, which corresponded most likely to an acclimation period of the microorganisms to the most refractory MTZ by-products. Then, from 288 h to the end of the culture, the TOC value decreased slightly to reach 84.6 % and 83.3 % for 1 h and 2 h respectively. Such behavior of microorganisms toward electrolyzed solutions, namely a rapid assimilation of the biodegradable part followed by an

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acclimation period and then an assimilation of a part of the remaining refractory compounds, was in agreement with other findings [38,40].

It should be observed that even if similar final mineralization yields (after 504 h) were obtained, higher mineralization yields were observed during the first 7 days in the case of 2 h electrolysis, in agreement with the higher biodegradability value, 0.46 and 0.7 for 1 and 2 h respectively (Fig.4). However, after 504 h, similar mineralization yields were obtained for 1 and 2 h of pretreatment.

Indeed, an additional pretreatment time of 1 h (2 h in total) did not affect the refractory product which remained after a 1 h pretreatment. The gain in terms of mineralization during biological treatment was not enhanced by the supplementary 1 h of electrolysis. It can be deduced from this result that the application of 1 h of electrolysis is more cost-effective and provides a similar percentage of mineralization. Similar result was obtained in the study of Ferrag-Siagh et al, with close mineralization yield values obtained after biological treatment of tetracycline for 2 and 4 h of electro-Fenton pretreatment despite a higher biodegradability after 4 h [40].

3.8. By-products monitoring during the biological treatment

After determining the appropriate electro-Fenton pretreatment time, LC-MS/MS analyzes were carried out to detect the electrolysis by-products after 1 h of pretreatment; the compounds 5, 7, 8, 9 and 10 given in the Table 2 were detected. These compounds constituted a part of the carbon source available for microorganisms during the biological treatment. After 504 h of biological treatment of 1 h electrolysis by-products, a detection of these compounds was carried out by LC-MS/MS in order to determine the behavior of the microorganisms with respect to these by-products. The results showed that the compounds 9 and 10 disappeared (data not shown), showing their assimilation or biotransformation by the microorganisms while the other by-products were still detectable, namely compounds 5, 7 and 8. However, the peak area corresponding to the compound 8 decreased (data not shown) demonstrating the ability of the microorganisms to biodegrade or biotransform at least partially this compound. Contrarily, compounds 5 and 7 were neither biodegraded nor biotransfomed; this may suggest that these compounds can be considered refractory and belongs to " Hard COD ". The behavior of the microorganisms with respect to the compounds detected can be explained by the influence of the substituted groups on the imidazole ring. In fact, the compounds' biodegradation depends

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on the interactions between the microbial enzymes and the substrate. The substituents on the aromatic ring significantly influence the probability of enzymatic attack on the substrate [45]. Then it can be supposed that the assimilation of compounds 9 and 10 can be due to the presence of hydroxyl groups as a substituent on their imidazole ring, since their presence improves the biodegradability of organic compounds [46,47]. The lower degradation of compound 8 can be attributed to the presence of a steric hindrance between the alcohol group OH and the acid group COOH. Several studies have described that the large volume and the steric effect of the substituents have a negative influence on the biodegradability [45,48]. However, the refractory character of the compounds 5 and 7 can be related to the presence of methyl group as substituent, since the presence of such group increases the persistence of the compounds [46–48].

4. Conclusion

A biological treatment of metronidazole was carried out with activated sludge showing no mineralization of MTZ; whereas 22 % of the MTZ was bio-transformed to 2-methyl-5-nitroimidazole-1-acetic acid (MAA), a by-product that has a phytotoxicity on seed garden cress higher than MTZ. This result showed the relevance of an electrochemical pre-treatment. The degradation of MTZ by electro-Fenton was carried out leading to an increase in biodegradability. From 1 h of EF electrolysis, the by-products formed can be considered as partially biodegradable (BOD_5/DCO =0.46); which was therefore considered as the optimum pretreatment time. After having verified the absence of biosorption of the electrolysis by-products on the wall of the microorganisms present in the activated sludge, a biological treatment of the by-products electrolyzed for 1 h was then realized. Identification of the electrolysis by-products of MTZ during electro-Fenton and the biological treatment were carried out. A possible mechanism of MTZ degradation was then proposed. From the identification of the residual by-products after biological treatment, some explanations concerning the influence of their chemical structure on biodegradation were suggested. The mineralization yield after 504 h of biological treatment with activated sludge of an electrolyzed solution was 84.6 %, namely 87.4 % of mineralization if both the pretreatment and the biological process were considered. These results show the efficiency of coupling an electro-Fenton process with a biological treatment with activated sludge for MTZ mineralization.

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Figures Captions

Fig. 1: Time-course of MTZ concentration (▲) and TOC (◆) during MTZ degradation by EF. Experimental conditions: C_0 100 mg.L $^{-1}$, 0.07 mA.cm^{-2}, [Fe (II)] = 0.1 mmol.L^{-1}, pH = 3, [Na_2SO_4] =0.05 mol.L^{-1}.

Fig. 2: Pathway proposed for the mineralization of MTZ by the electro-Fenton process.

Fig. 3: Time-course of the concentration of inorganic species during MTZ degradation by EF. Experimental conditions: C_0 = 100 mg.L $^{-1}$, 0.07 mA.cm^{-2}, [Fe (II)] = 0.1 mmol.L^{-1}, pH =3, [Na_2SO_4] =0.05 mol.L^{-1}.

Fig. 4: Evolution of the BOD_5 on COD ratio during electrolysis. Experimental conditions: C_0 = 100 mg.L $^{-1}$, 0.07 mA.cm^{-2}, [Fe (II)] = 0.1 mmol.L^{-1}, pH = 3, [Na_2SO_4] =0.05 mol.L^{-1}.

Fig. 5: Time-courses of adsorption on living (L) and dead (D) cells sludge, at 25 °C, initial pH 7 of 100 mg.L^{-1} non-treated MTZ (P) and MTZ solutions electrolyzed for 1 h (E) at pH 3, 0.07 mA.cm^{-2} , [Na_2SO_4] = 0.05mol.L^{-1} and [Fe^{2+}] = 0.1 mmol.L^{-1}.

Fig. 6: Time-courses of the MTZ concentrations (▲) and TOC (◆) values during activated sludge culture on non-pretreated MTZ solution. C_0 = 100 mg.L $^{-1}$ [Activated sludge]= 0.5g.L^{-1} , 200 rpm , 25°C , pH = 7

Fig. 7: HPLC chromatogram for MTZ degradation and by-product formation during the direct biological treatment.

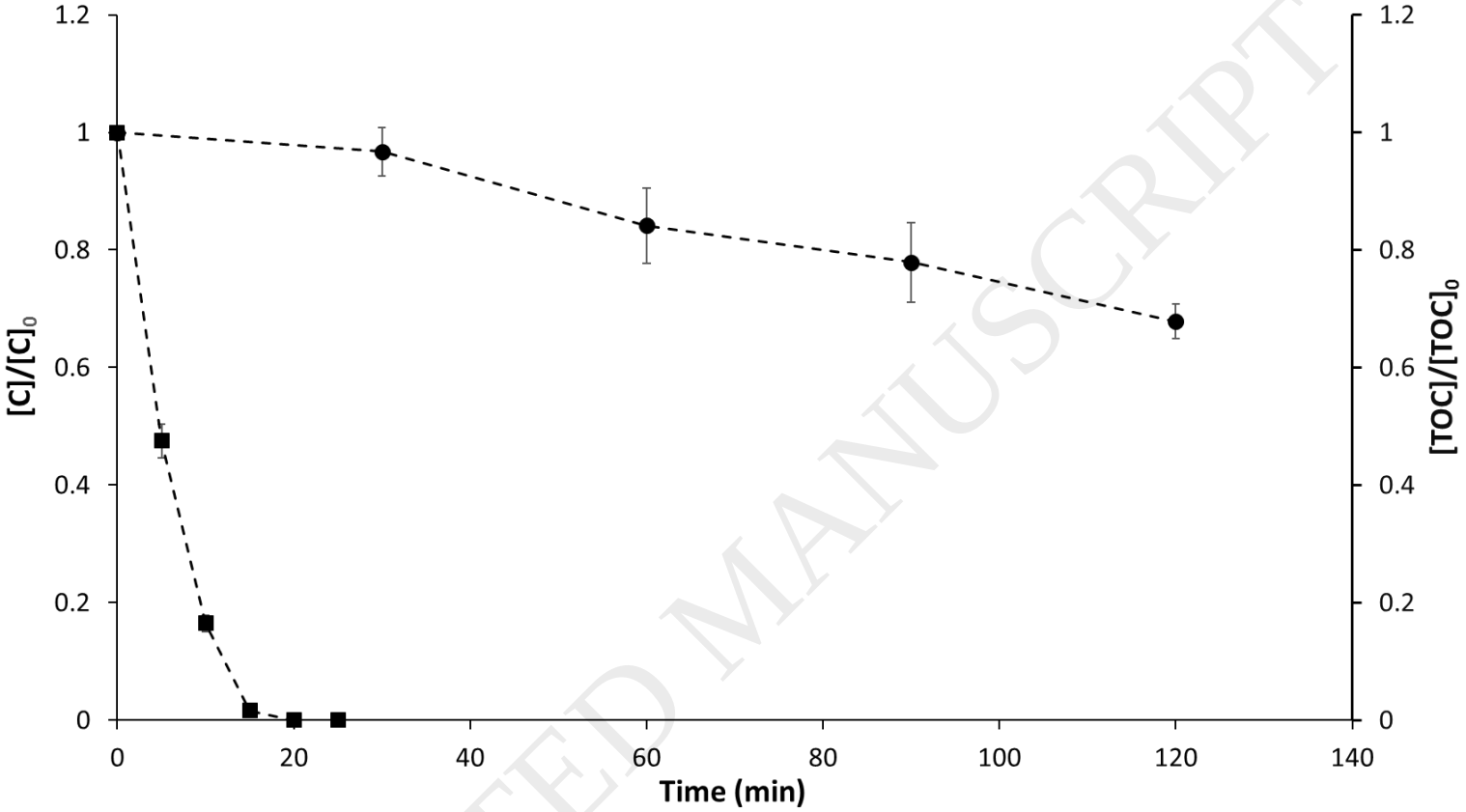
Fig. 8: The phytotoxicity of the main MTZ molecule and the MAA byproducts formed during direct biological treatment.

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Fig. 9: Time-courses of the TOC values during activated sludge culture on pretreated MTZ solutions for 1 h (◆) and 2 h (▲). [Activated sludge] = 0.5g.L^{-1} , 200 rpm , 25°C , pH = 7

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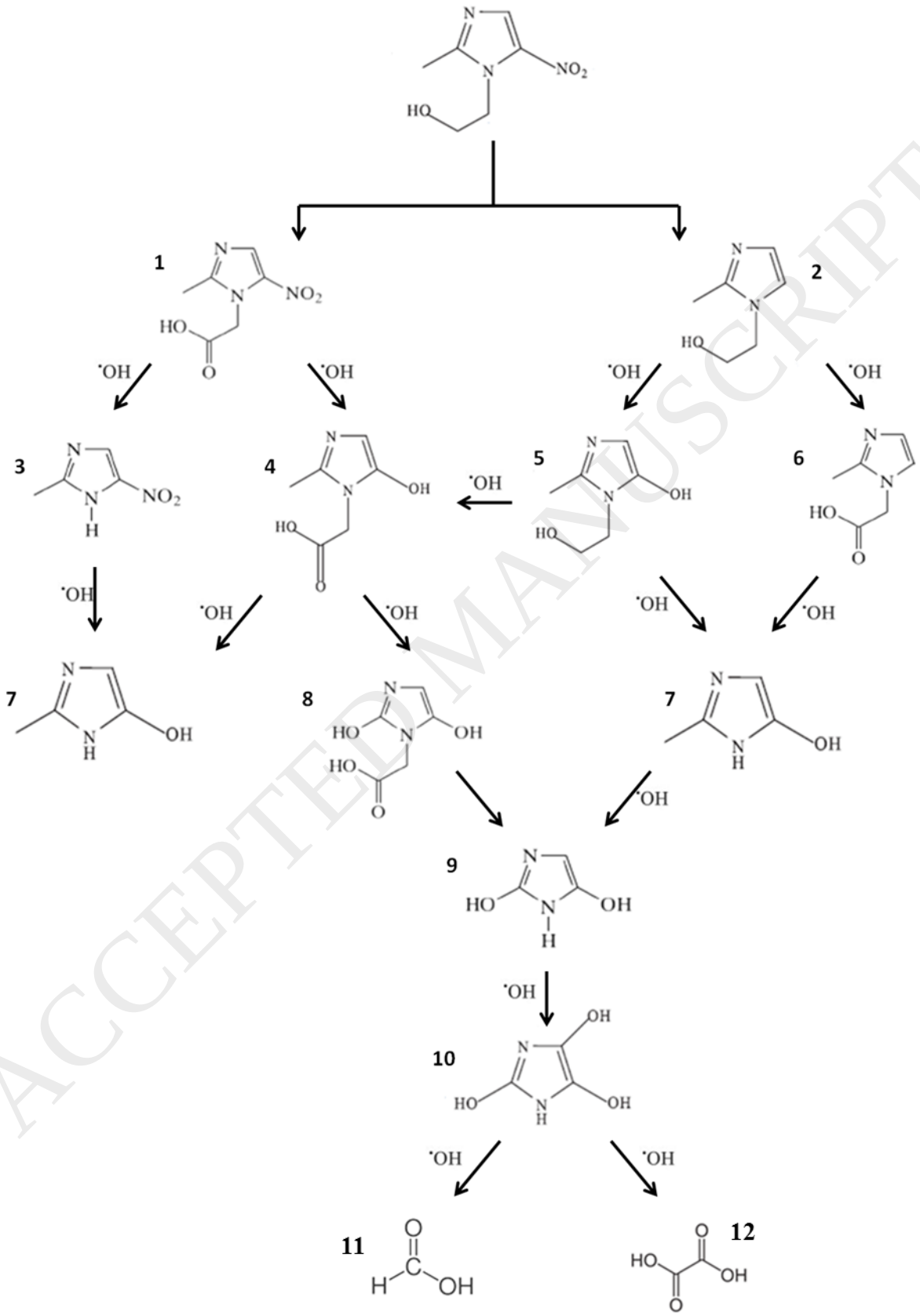


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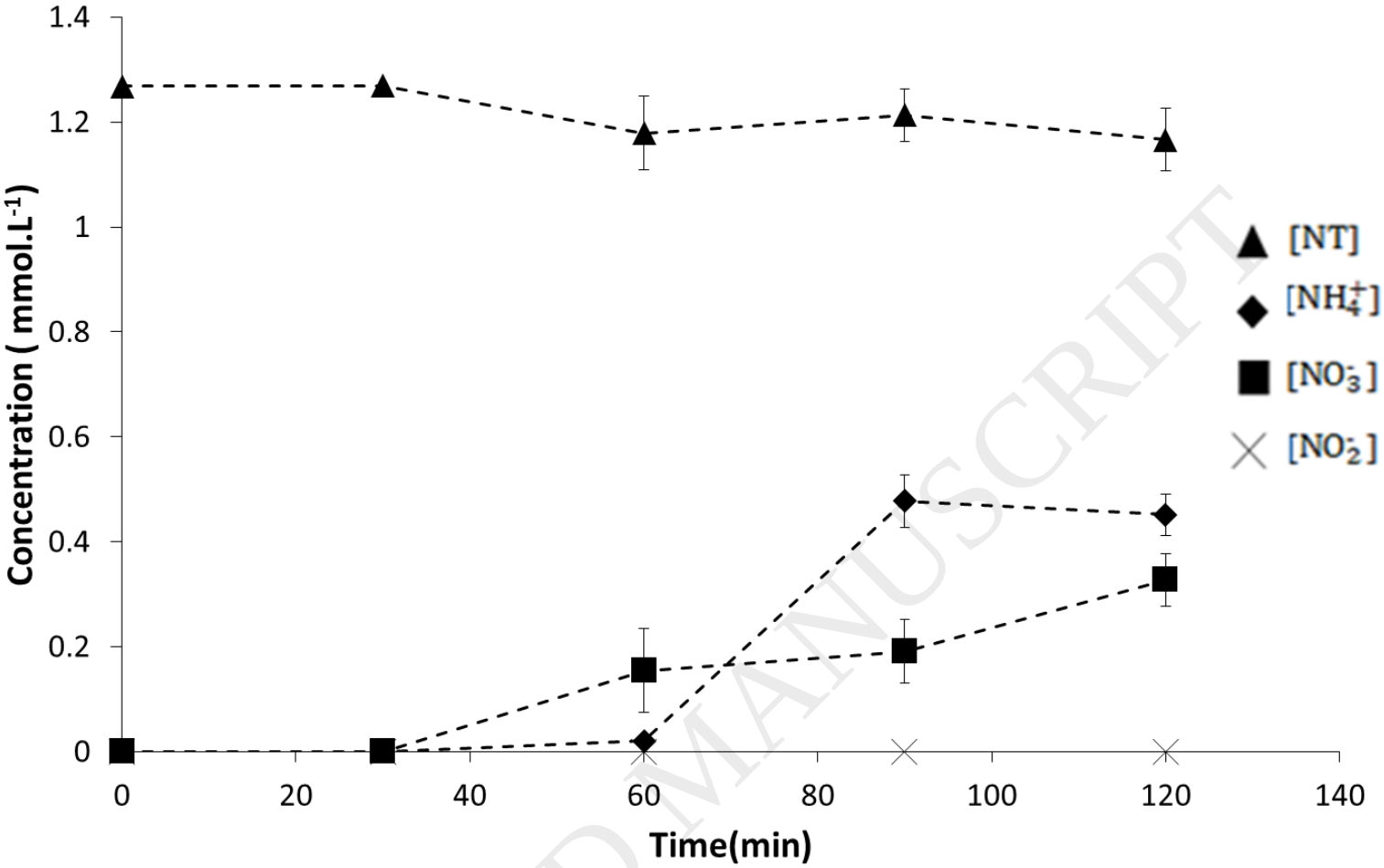
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Metronidazole

Mw171.15 g.mol^{-1}

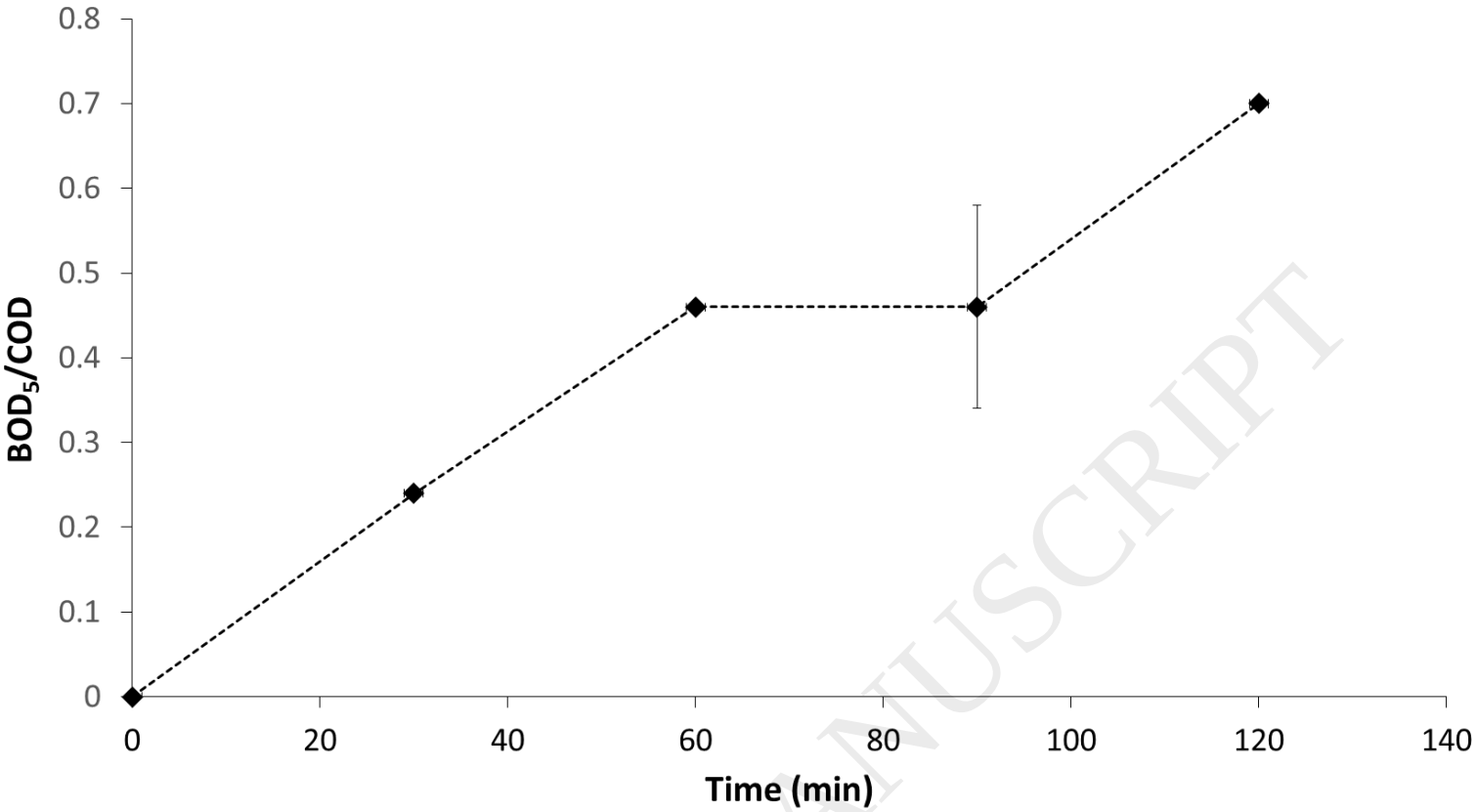


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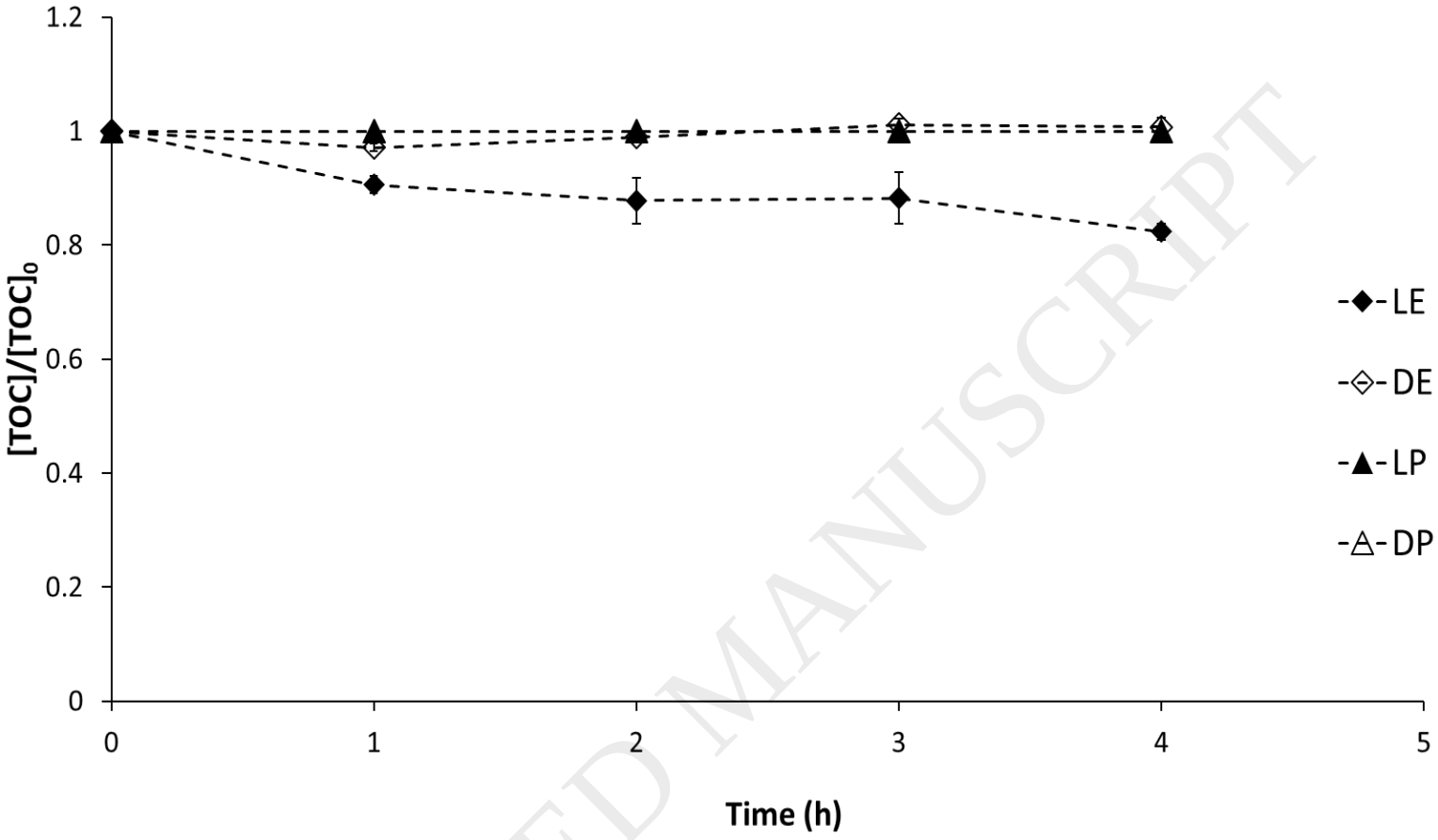
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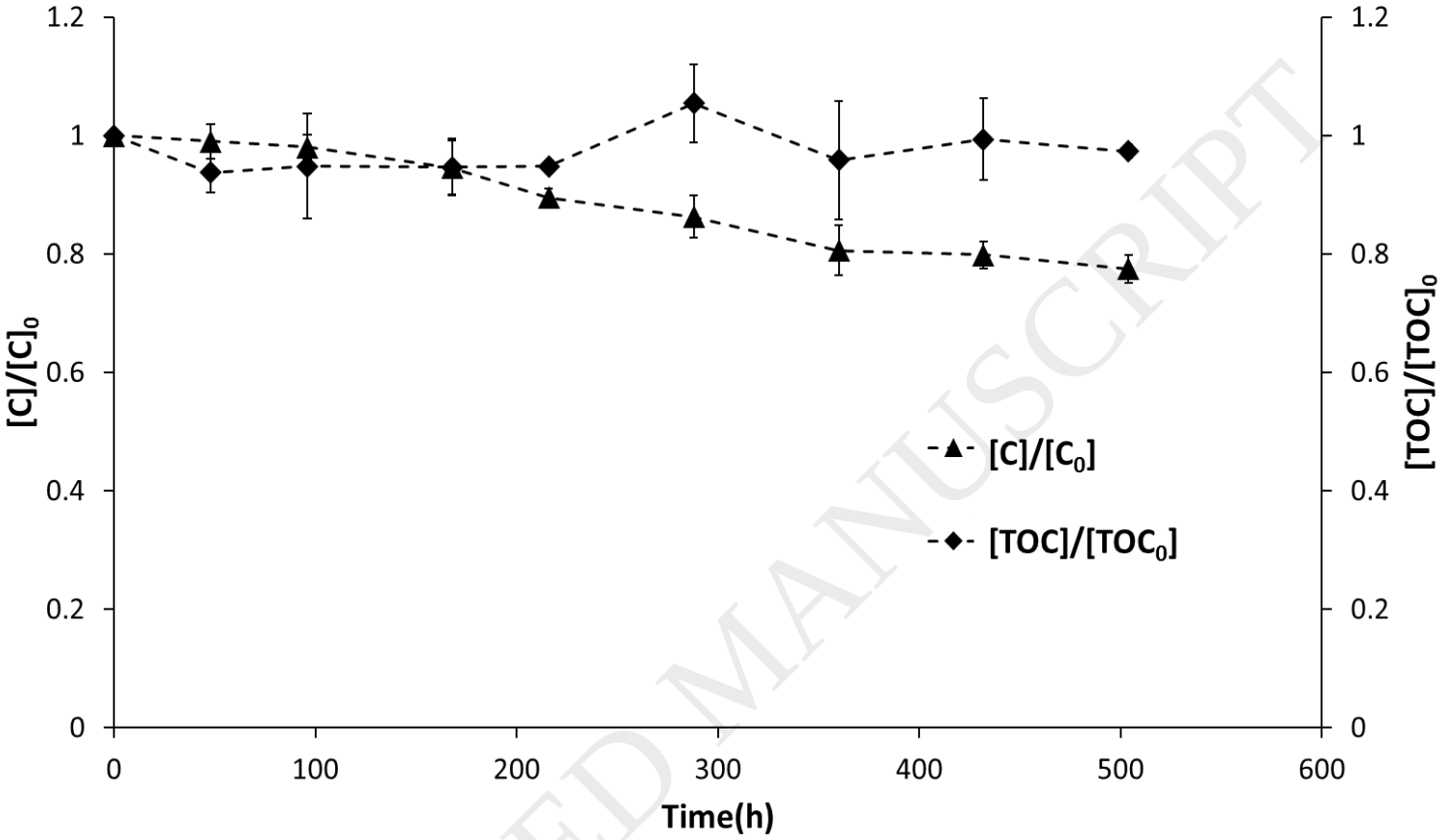
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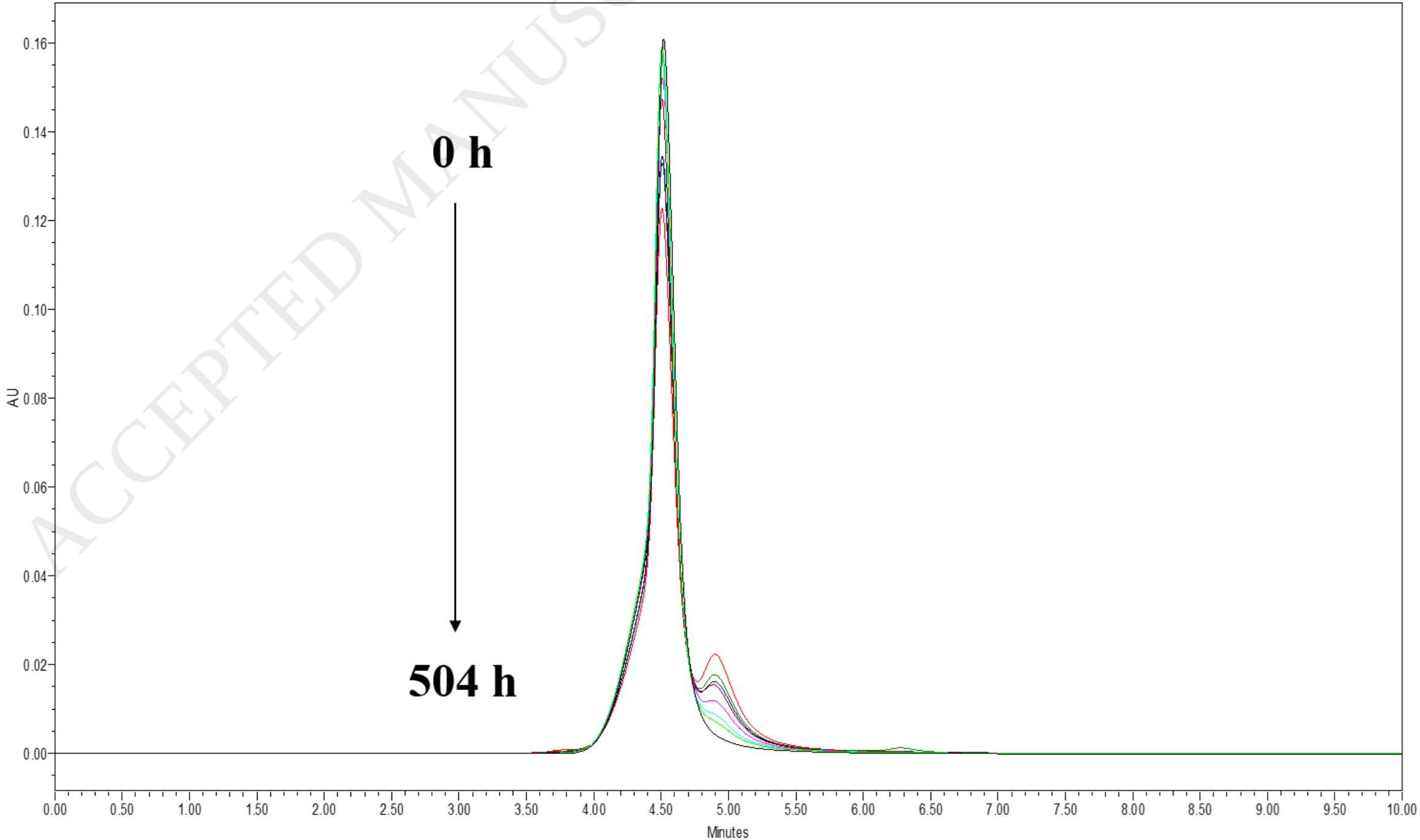


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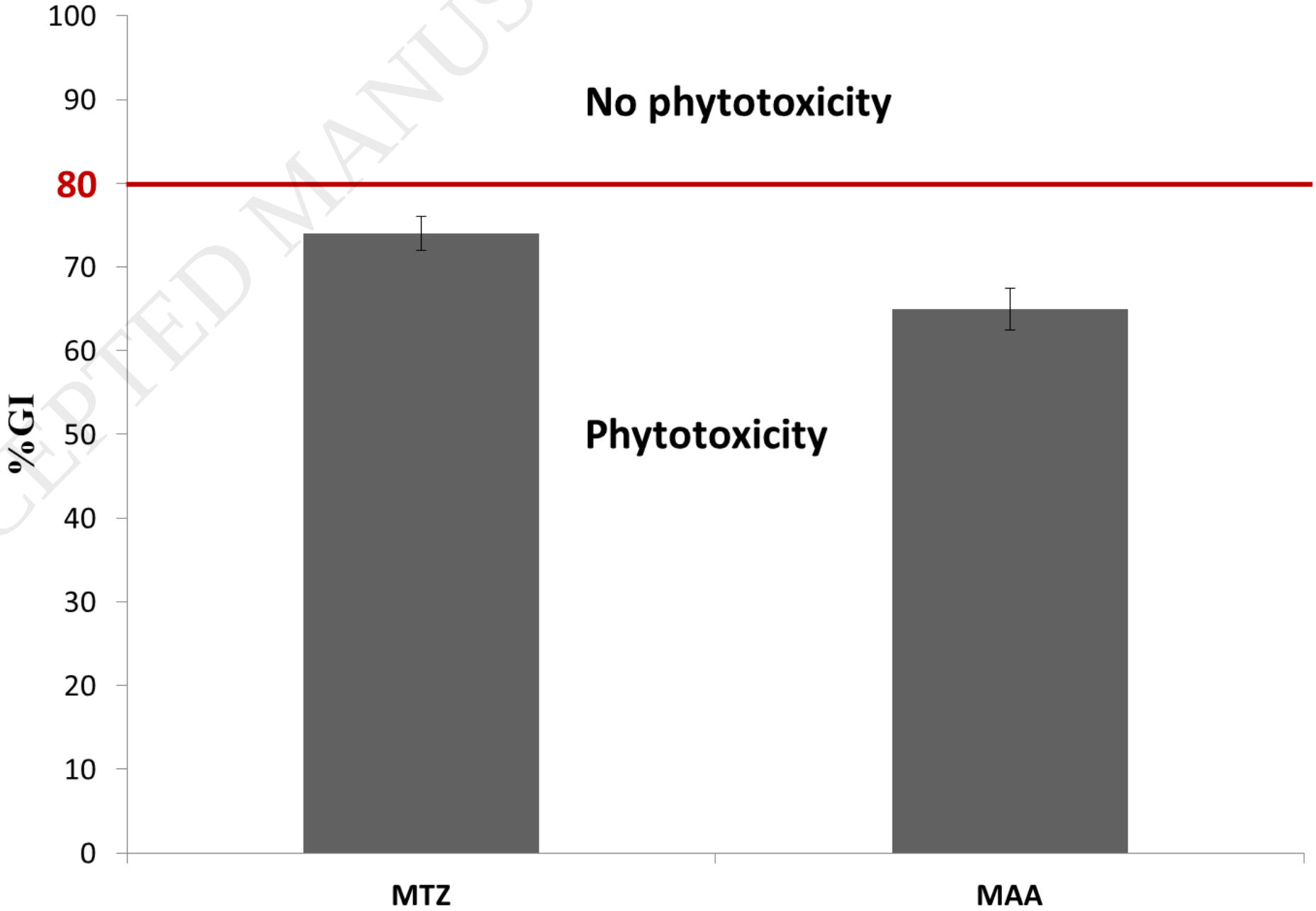
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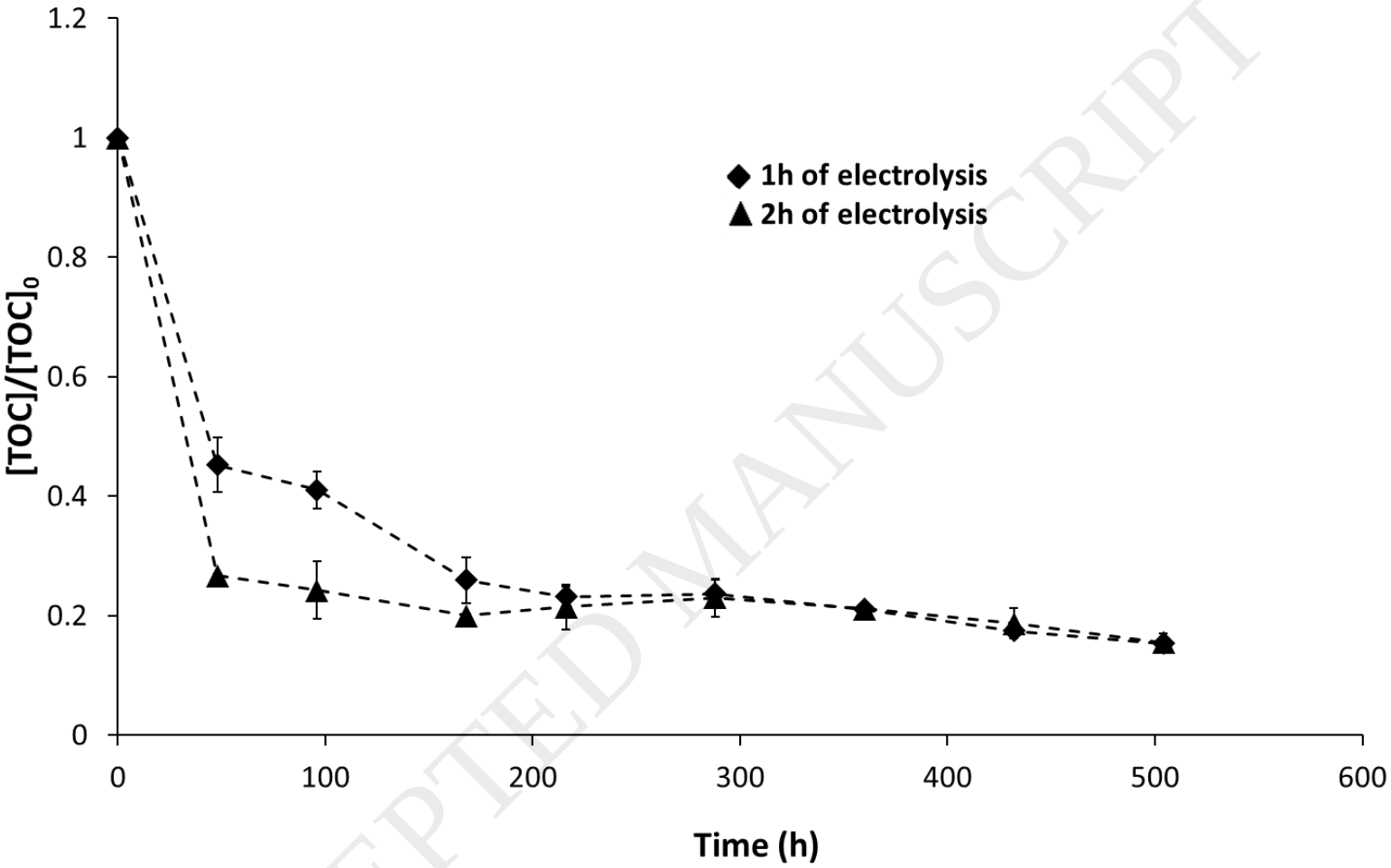


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Table Legends

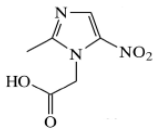
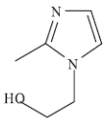
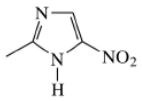
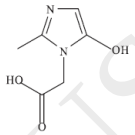
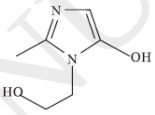
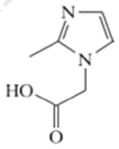
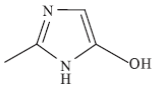
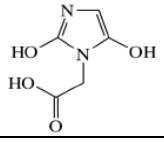
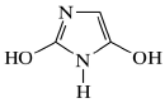
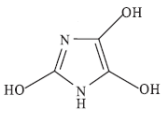
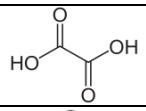
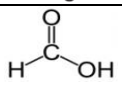
Table 1: The reagents used for the BOD test

	Reagents	**Concentration ($g.L^{-1}$)**
Mineral basis	$MgSO_4.7H_2O$	22.5
	$CaCl_2$	27.5
	$FeCl_3$	0.15
	NH_4Cl	2.0
Buffer solution	Na_2HPO_4	6.80
	KH_2PO_4	2.80
Nitrification inhibitor	N-allylthiourea	0.5

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Table 2: Intermediates products formed during the electrolytic degradation of metronidazole

Compound	Name	Chemical structure	Molar mass ($g.mol^{-1}$)
1	(2-methyl-5-nitroimidazol-1-yl) acetic acid		185
2	1-hydroxyethyl 2-methyl imidazole		126
3	2-methyl 5-nitroimidazole		127
4	(5-hydroxy-2-methyl imidazol-1-yl) acetic acid		156
5	1-hydroxyethyl 2-methyl 5-hydroxyimidazol		142
6	(2-methylimidazol-1-yl) acetic acid		140
7	5-hydroxy 2-methyl-imidazole		98
8	2,5-dihydroxyimidazol -1-yl- acetic acid		158
9	2,5 dihydroxy imidazole		100
10	2,3,5-trihydroxyimidazole		116
11	Oxalic acid		90
12	Formic acid		46